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Isolation, Molecular Detection and Phylogenetic analysis of Avipox Virus obtained from Pigeon

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Abstract

Dried scab material obtained from pigeon showing cutaneous form of avipox infection was inoculated in chorio-allantoic membrane (CAM) of 11-day old embryonated chicken egg. After an incubation of five days, CAM showed mild pock lesions. This CAM tissue was subjected to DNA isolation. Molecular confirmation of avipox virus was done by PCR amplification of P4b gene, which amplified at an expected product size of 578 bp. The amplified P4b gene was sequenced and phylogenetic analysis was done along with P4b gene sequences of other avipox viruses. Phylogenetic analysis showed that this avipox virus obtained from pigeon was grouped under sub-clade A2 along with other pigeonpox viruses. This confirmed that the causative agent of the avipox infection observed in pigeon was *pigeonpox virus.*

Introduction

Avipox is a contagious viral disease that occurs in various species of birds across the world (Bolte *et al*., 1999). The disease is characterized into two forms based on the lesions formed during disease pathogenesis: cutaneous form and diphtheritic form. Cutaneous form is characterized by formation of wart-like nodules around the eyes, beak and nostrils. These nodular lesions harden and form scabs. Diphtheritic form is characterized by formation of proliferative nodular lesions in mucous membrane of mouth,larynx, pharynx, trachea and oesophagus (Tripathy and Reed, 2003). These nodular lesions cause difficulty in breathing and eating, leading to morbidity and mortality. The disease is transmitted either by direct contact with the infected bird, its infectious particles or by arthropod bite. Diagnosis of avipox disease was based significantly on clinical signs, which was confirmed by virus isolation, histopathological examination, electron microscopy and restriction-fragment length polymorphism. Later on , confirmation of avipox disease by molecular

methods was developed. This was done by PCR amplification of the highly conserved core virion protein (P4b) gene (Lee and Lee, 1997).

Avipox is caused by *Avipox virus* (APV) genus of *Chordopoxvirinae* subfamily of *Poxviridae* family. The genus APV encompasses ten recognized virus species based on the bird-species affected. They include *Canarypox virus, Fowlpox virus, Juncopox virus, Mynahpox virus, Pigeonpox virus, Psittacinepox virus, Quailpox virus, Sparrowpox virus, Starlingpox virus* and *Turkeypoxvirus.* Based on phylogenetic analysis, many species of APV have been identified from various bird species (Jarmin *et al*., 2006; Gyuranecz *et al*., 2013). Phylogenetic analysis of APV is based on the highly conserved core virion protein (P4b) gene, that shares 64.2% amino acid identity among the APVs. Based on P4b gene based phylogenetic analysis, APVs are classified into three clades (Jarmin *et al*., 2006): Clade A – fowlpox-like virus, consisting of seven sub-clades A1 to A7; Clade B – Canarypox-like virus consisting of four sub-clades B1 to B4 and Clade C – Psittacinepox-like virus. Apart from these three clades, two more clades D and E were also reported (Manoralla *et al*., 2010; Banyai *et al*., 2015). Thus, P4b gene was used as molecular detection tool for confirmation of avipox disease and was used in phylogenetic analysis to ascertain the virus species.

In this study, an avipox virus obtained from a pigeon showing cutaneous pock lesions was isolated and was subjected to molecular confirmation and phylogenetic analysis based on P4b gene.

Materials and Methods

Viral isolation

The dried scab was collected from the avipox infection suspected pigeon. The scab material was homogenized and re-suspended in phosphate buffered saline containing 50 IU/ml Pencillin and 50 µg/ml Streptomycin.The debris from suspension was removed by centrifugation at 3000 rpm for 10 minutes and the supernatant was inoculated onto chorio-allantoic membrane (CAM) of 11-day old embryonic chicken egg (ECE) and incubated at 37°C for 5 days (Tripathy and Reed, 2003).

DNA isolation and P4b gene amplification

The CAM tissue was subjected to DNA isolation using QIAampDNA investigator kit (Cat#56504, Qiagen, Germany) following manufacturer's instructions. The isolated DNA was amplified by polymerase chain reaction using primers specific for P4b gene (Binns *et al*., 1989): P4b forward primer: 5'-CAGCAGGTGCTAAACAACAA-3' and P4b reverse primer: 5'-CGGTAGCTTAACGCCGAATA-3'. The reaction mixture comprised of 5 µl Primestar master mix (Cat#R045A, Clonetech, USA), 10 pmol of each primer, 50 ng of DNA to a final reaction volume of 50 µl. The reaction cycle involved an initial denaturation at 98°C/3 min followed by 30 cycles of denaturation at 98°C/30 sec, annealing at 53°C/30 sec and extension at 72°C/1 min for 30 cycles with a final extension at 72°C/5 min. The resultant PCR product was separated by agarose gel electrophoresis and was purified using Nucleospin Gelâ and PCR cleanup kit (Cat#740609.50, Machery-Nagel, Germany).

Sequencing and phylogenetic analysis of P4b gene

The eluted PCR product of P4b gene was sequenced and analysed by BLAST. Phylogenetic analysis of the PGPV under study was performed by neighbor-joining method with a bootstrap value of 1000 along with avipox virus P4b gene sequences retrieved from GenBank database using MEGA 7.0 software (Kumar *et al*., 2016).

Results and Discussion

The dried scab material obtained from the pigeon suspected for avipox infection was isolated on CAM of ECE. The CAM tissue was hemorrhagic and slightly thickened in the first two passages, whereas on the third passage mild pock lesions (Figure 1) were observed (Gilhare *et al*., 2015). The CAM tissue was positive for P4b gene PCR amplification, which yielded an expected product size of 578 bp (Figure 2). The amplified P4b gene was sequenced and analyzed by BLAST, which showed 100% similarity with *pigeonpox virus* isolates from South Africa, Germany, Egypt and India. Upon P4b gene based phylogenetic analysis, the isolate under study was grouped under sub-clade A2 (Figure 3), which is specific for *pigeonpox virus* (Jarmin *et al*., 2006). Thus,

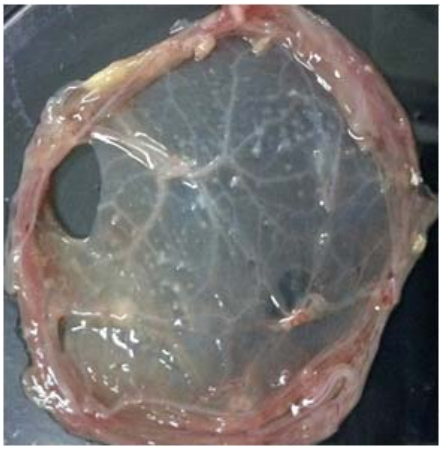

Figure 1 : CAM tissue showing mild pock lesions in third passage

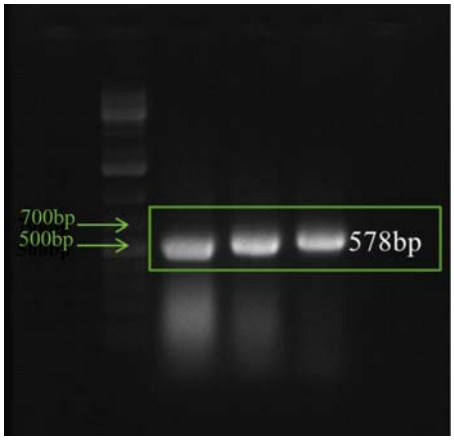

Figure 2 : Agarose gel electrophoresis showing 578bp amplification of P4b gene

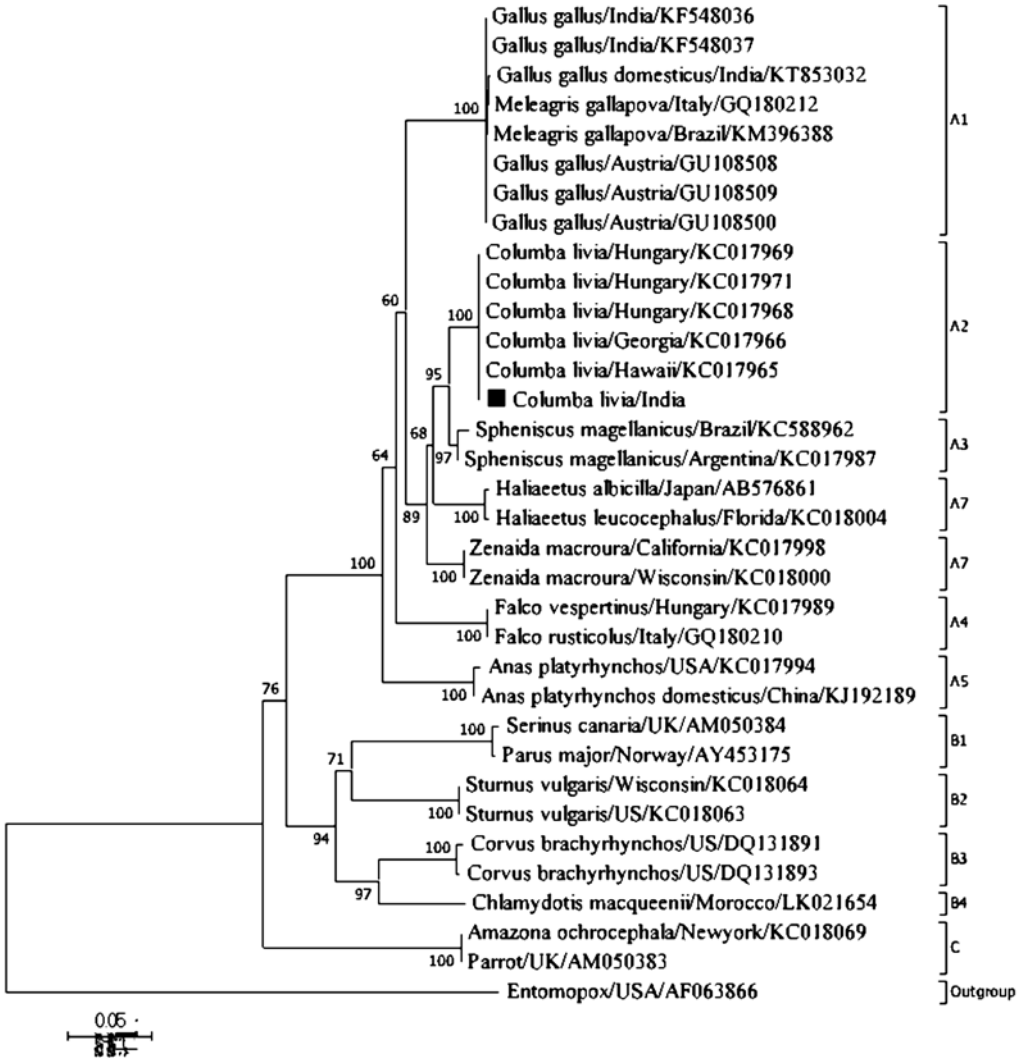

Fig. 3 : P4b gene based phylogenetic tree using Neighbour-joining method with 1000 bootstrap replicates

the pigeon suspected of avipox infection was affected with *pigeonpox virus* species.

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Conflict of interest :

The authors declare that they have no conflict of interest.

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